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13. Abstract (Maximum 200 Words) *(abstract should contain no proprietary or confidential information)*
p27 is a CDK inhibitor that controls the G1/S transition. Low or absent expression of p27 has been observed in many malignant tumors including breast cancer. SCF^{SKP2} is a ubiquitin E3 ligase that interacts and targets p27 for ubiquitin-dependent proteolysis. One aim of the proposal is to determine whether there is an inverse relationship between SCF^{SKP2} and p27 in breast cancer and the experiments are in progress. Another aim is to determine the domain on SKP2 that binds to p27. We have determined the carboxy-terminal region of SKP2 is required for binds to phosphorylated p27. The critical aim is to determine whether inhibition of SCF^{SKP2} in breast cancer cells causes cell cycle arrest. Our studies suggest that inhibition of SCF^{SKP2} in breast cancer and other cancer cells causes accumulation of p27 and G1 cell cycle block. Thus SCF^{SKP2} may be used as a target for breast cancer therapy.

14. SUBJECT TERMS:
p27, ubiquitin E3 ligase, SCF^{SKP2}, cell cycle regulation, CDKs

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Annual report (July 1, 2001-June 30, 2002):

Introduction:

The objective of this research is to investigate whether inhibition of the CDK inhibitor p27^{Kip1} degradation pathway could be used for breast cancer therapy. p27 coordinates the cell cycle by binding and thus inhibiting the cyclin E/CDK2 kinase activity, preventing the unscheduled entry of S phase. In the cell cycle, p27 is controlled primarily by ubiquitin-dependent proteolysis [1]. The levels of p27 are high in G0 and G1 cells, and become abruptly proteolyzed at late G1, allowing S phase entry. To understand p27 proteolysis, it is critical to determine the mechanism for its ubiquitin-dependent degradation. Ubiquitin-mediated protein degradation requires the activation of ubiquitin by forming a covalent thio-ester linkage between ubiquitin and the ubiquitin E1 activation enzyme in the presence of ATP. The activated ubiquitin is then transferred onto the E2 conjugating enzyme. In most cases, the substrate specificity of ubiquitination is mediated through the ubiquitin E3 ligases, which bridge the interaction between the substrates and E2 enzymes [2].

We have previously isolated SCF^{SKP2} (SKP1, CUL1/CDC53, F-box protein-SKP2) ubiquitin E3 ligase complex and have shown p27 as its critical substrate [3]. Specifically, SKP2, an F-box protein, binds and targets the threonine 187-phosphorylated p27 for ubiquitin-dependent degradation. In many cancers, low or absent expression of p27 is associated with malignant cancers, including breast and prostate cancers [4]. Since expression of SKP2 is sufficient to down-regulate p27 and it has been shown that SKP2 is over-expressed in cancer cell lines and many cancer samples [5-7], it is proposed that inhibition of SCF^{SKP2} activity may cause accumulation of p27 and cell cycle arrest in breast cancer cells. The major aim of the grant is to explore the use of SCF^{SKP2} as the target for breast cancer therapy.

Body:

Task 1: To determine whether high levels of SCF^{SKP2} is inversely correlated with p27.

a. Affinity purification of anti-SKP2, p27, SKP1, and CUL-1 antibodies. We have accomplished the affinity purification anti-SKP2, p27, SKP1 and CUL-1 polyclonal antibodies and examined their ability to immunoprecipitation and Western blotting in cell lysates. Judged from our data it was confirmed that all the affinity purified antibodies are active.

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b. We have ordered breast cancer section slides for immunostaining of the affinity purified antibodies. One problem we have encountered was that the staining had high background. We are using various alternative ways, such as change of blocking reagents, etc., to lower the background.

Task 2: To systematically analyze the interactions between SKP2 and the phosphorylated p27.

a. Define SKP2 binding region to phosphorylated p27 by deletion.
We have made deletions of SKP2 from both amino and carboxy terminal ends and measured the binding region of SKP2 to phosphorylated p27 at threonine 187. Our results suggest that the carboxy terminal region of SKP2 is required for phosphorylated p27 binding (Figure 1).
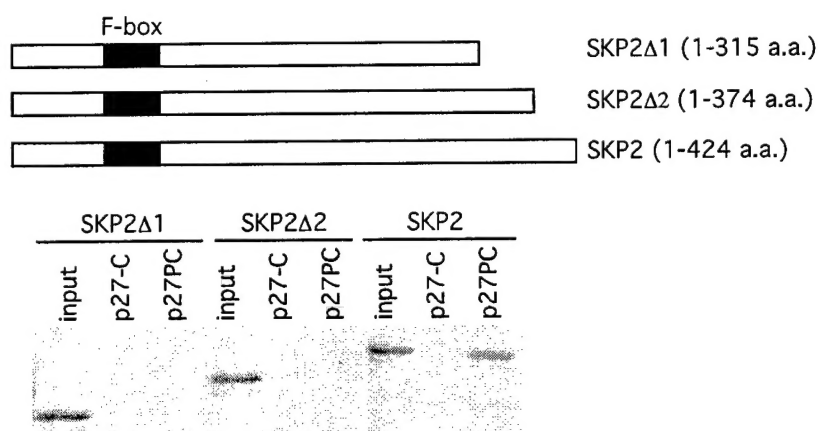

Figure 1. The carboxy terminal region of SKP2 is required for binding to the phosphorylated threonine 187 in p27. In vitro translated SKP2 mutants and wild-type SKP2 (input) were incubated with p27 carboxy terminal peptides without (p27C) or with phospho-threonine 187 (p27CP).

b. Our data suggest that the carboxy terminal region of p27 is involved in the SKP2 binding (Figure 1).

c.Making the fine mutational analysis of SKP2 for p27 binding: Still in progress. However, it appears that the entire carboxy half of SKP2 down-stream of the F-box is required for p27 binding.

d. Examine p27 peptide for inhibition of p27 degradation: Still in progress.

Task 3: To determine whether disruption of SKP2-p27 interaction inhibits proliferation of breast cancer cells.

a. Establish the retrovirus assays for MCF-7 breast cancer cell line for retrovirus delivery.

We have tried to establish the Eco-receptor which serves as retrovirus receptor for virus infection on MCF-7 cells. We could establish Eco-receptor on MCF-7 cells. However, the cells quickly lost their receptors and the remaining population expresses very low concentration of receptors which prevent an efficient infection. We are trying to use Bing cells and pan-retrovirus package systems to infect the MCF-7 cells.

b-d. Effect of SCF^{SKP2} inhibition on p27 and cell cycle.

We have tried to express the SKP2 carboxy terminal or SKP2 dominant negative domain which contain a small deletion in the F-box region. These mutants are capable of binding to p27 but are ineffective to degrade p27. The effect of these mutants were analyzed and it appears that SKP2 mutants had small inhibitory effect on cell growth. We are using the newly developed siRNA technique to determine whether silencing of SKP2 is sufficient to block cell growth. Our results indicated that ablation of SKP2 by siRNA is effective which leads to the accumulation of p27 and cell cycle arrest (Figure 2). We also used a newly isolated CUL-1 associated protein, p120^{CAND1}, which we found to act as an inhibitor of SCF^{SKP2} complex, for the effect on p27. Our data suggest that CAND1 can block the activity of SCF^{SKP2} in both cells and in vitro cell extract for p27 degradation (Figure 3) [8]. Thus these new data suggest inhibition of p27 degradation can inhibit cell growth.
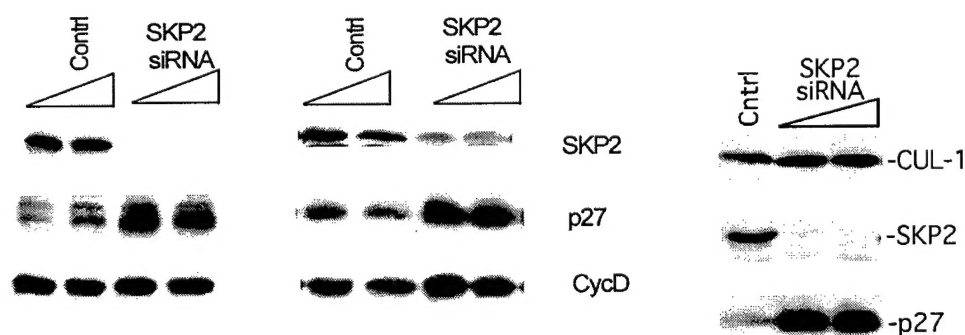

Figure 2. Silencing of SKP2 expression in breast cancer cells MDA-MB-435 (left), MCF-7 (middle), and cervical carcinoma HeLa cells (right) causes accumulation of p27.
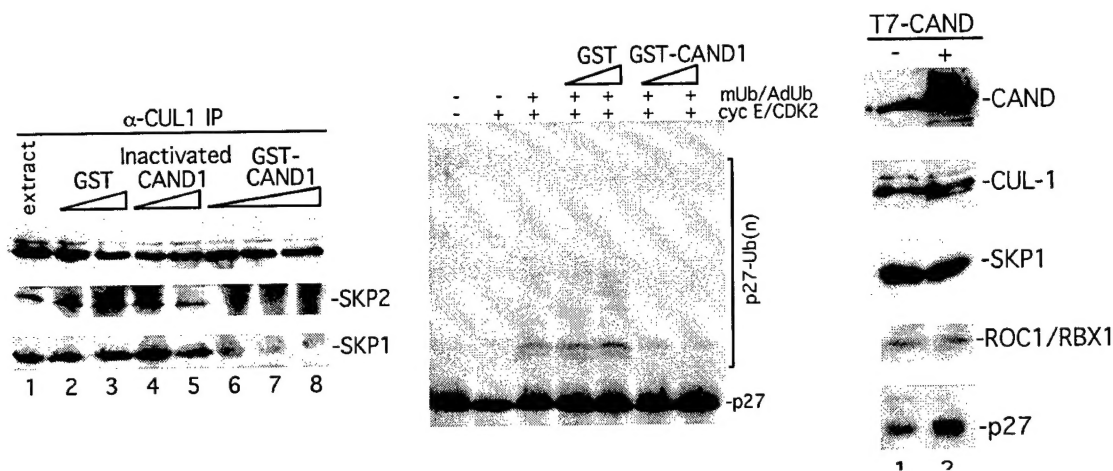
Figure 3. Addition of SCF inhibitor p120^{CAND1} inhibits SKP1 and SKP2 association and p27 polyubiquitination in HeLa extract. Expression of CAND1 in HEK 293 cells causes p27 accumulation.
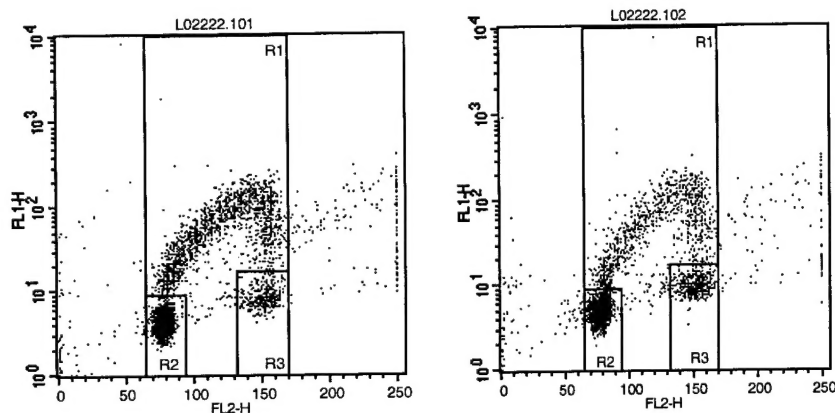
Figure 4. Silencing of SKP2 causes G1 cell cycle arrest. Human U87MG cells were treated with 150 nM siRNA against either control (luciferase, left) or SKP2 (right) for 48 hrs before harvesting for FACS analysis. The silencing of SKP2 causes increase of G1 (from 57% to 65%), reduction of S (from 34% to 23%), and slight increase of G2/M population (from 9% to 12%).

Key research accomplishments:

We show that inhibition of p27 degradation by blocking the activities of SCF^{SKP2} ubiquitin E3 ligase can cause cell growth arrest.

Reportable outcome:

1) Zheng, J., Yang, X., Harrell, J., Ryzhikov, S., Shim, E., Lykke-Andersen, K., Wei, N., Sun, H., Kobayashi, R., and Zhang, H. (2002). CAND1 Binds to

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Unneddylated CUL1 and Regulates the Formation of SCF Ubiquitin E3 Ligase Complex. *Molecular Cell* 10, 1519-1526.

2) Manuscript in preparation.

Conclusion:

we found that inhibition of SCF^{SKP2} complex is able to block p27 degradation and arrest cell cycle. Thus blocking p27 degradation provides a novel way for breast cancer therapy.

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CAND1 Binds to Unneddylated CUL1 and Regulates the Formation of SCF Ubiquitin E3 Ligase Complex

Short Article

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Summary

The SCF ubiquitin E3 ligase regulates ubiquitin-dependent proteolysis of many regulatory proteins such as p27[Kip1], IκB, and β-catenin. We report the isolation of a CUL1 binding protein, p120[CAND1]. We found the majority of CUL1 is in a complex with CAND1 and ROC1 independent of SKP1 and F box protein SKP2. Both in vivo and in vitro, CAND1 prevents the binding of SKP1 and SKP2 to CUL1 while dissociation of CAND1 from CUL1 promotes the reverse reaction. Neddylation of CUL1 or the presence of SKP1 and ATP causes CAND1 dissociation. Our data suggest that CAND1 regulates the formation of the SCF complex, and its dissociation from CUL1 is coupled with the incorporation of F box proteins into the SCF complex, causing their destabilization.

Introduction

Cullin-1 (CUL1) is an essential component of the SCF (SKP1, CUL1/CDC53, F box proteins) ubiquitin E3 ligase complex that controls the protein levels of many regulatory proteins such as β-catenin, IκB, and p27[Kip1] (Amati and Vlach, 1999; Maniatis, 1999). CUL1 binds to SKP1, which in turn interacts with the substrate-targeting subunit, the F box proteins (Bai et al., 1996). CUL1 also associates with the RING finger protein ROC1 (also called RBX1 or HRT1) which links SCF to the ubiquitin-conjugating enzyme E2 and the activating enzyme E1 for the ubiquitin transfer reaction (Deshaies, 1999). CUL1 belongs to the cullin family consisting of at least six members (CUL1 to CUL6). All cullin-containing complexes appear to act as ubiquitin E3 ligases (Deshaies, 1999). For example, CUL2 forms a complex with the von Hippel-Lindau tumor suppressor (VHL) and elongin B and C, and regulates the stability of the hypoxia-inducible transcription factor HIF in response to oxygen levels (Ohh et al., 2002). CUL5 is involved in the degradation of p53 mediated by adenovirus E4orf6 and E1B55K proteins (Querido et al., 2001).

Despite the importance of cullins in controlling many essential biological processes, the mechanism that regulates the cullin-containing ubiquitin E3 ligases remains unclear. In SCF, the F box proteins are short-lived proteins that undergo CUL1/SKP1-dependent degradation (Wirbelauer et al., 2000; Zhou and Howley, 1998). Deletion of the F box region abolishes the binding of F box proteins to SKP1 and CUL1, and consequently increases the stability of F box proteins. This substrate-independent proteolysis of F box proteins is likely the result of autoubiquitination by the ubiquitin E2 and E1 enzymes through a CUL1/SKP1-dependent mechanism.

The carboxy-terminal ends of cullins are often covalently modified by a ubiquitin-like protein, NEDD8/RUB1, and this modification appears to associate with active E3 ligases (Hochstrasser, 2000). Like ubiquitin modification, neddylation requires E1 (APP-BP1 and UBA3)-activating and E2 (UBC12)-conjugating enzymes (Hochstrasser, 2000). Recently, the COP9 signalosome complex (CSN) was found to possess a specific isopeptidase activity to deneddylate cullins (Lyapina et al., 2001). CSN was originally identified in *Arabidopsis* and regulates photomorphogenesis (Wei and Deng, 1999). It is composed of eight subunits (CSN1-8) and is homologous to the lid complex of the 26S proteasome. The neddylation pathway is highly conserved from yeast to human and is essential in many organisms (Hochstrasser, 2000). Recent studies show that neddylation of CUL1 or CUL2 is required for the polyubiquitination of p27[KIP1], IκB, or HIF prior to proteolysis (Ohh et al., 2002; Podust et al., 2000; Read et al., 2000). However, the exact function of neddylation is not well understood.

The cell cycle is controlled by the cyclin-dependent kinases (CDKs) (Sherr, 1996). In mammals, the ubiquitin-dependent proteolysis of the CDK inhibitor p27[KIP1] triggers entry into S phase (Amati and Vlach, 1999). The proteolysis of p27 is regulated by the SCF[SKP2] ubiquitin E3 ligase. In particular, SKP2, an F box protein, binds and targets p27 for polyubiquitination and subsequent proteolysis. We are interested in the regulation of the SCF[SKP2] ubiquitin E3 ligase. Here, we report our finding that the SCF ligase is regulated by p120[CAND1], a protein we initially isolated as a CUL1 binding protein.

Results

Isolation of p120 as a CUL1-Associated Protein

To identify potential regulators of SCF, CUL1 complexes were immunoprecipitated from lysates of ^{35}S-methionine-labeled HeLa cells. We found that the majority of CUL1 was specifically associated with two proteins of 25 and 120 kDa, p25 and p120 (Figure 1A). p120 was isolated using anti-CUL1 immunoaffinity chromatography and was subjected to protein sequencing. The amino acid sequences of two derivative peptides were found to match the protein sequences of rat and human TIP120A, a protein previously isolated as a GST-TBP-interacting protein in HeLa extract (Yogosawa et al., 1996).

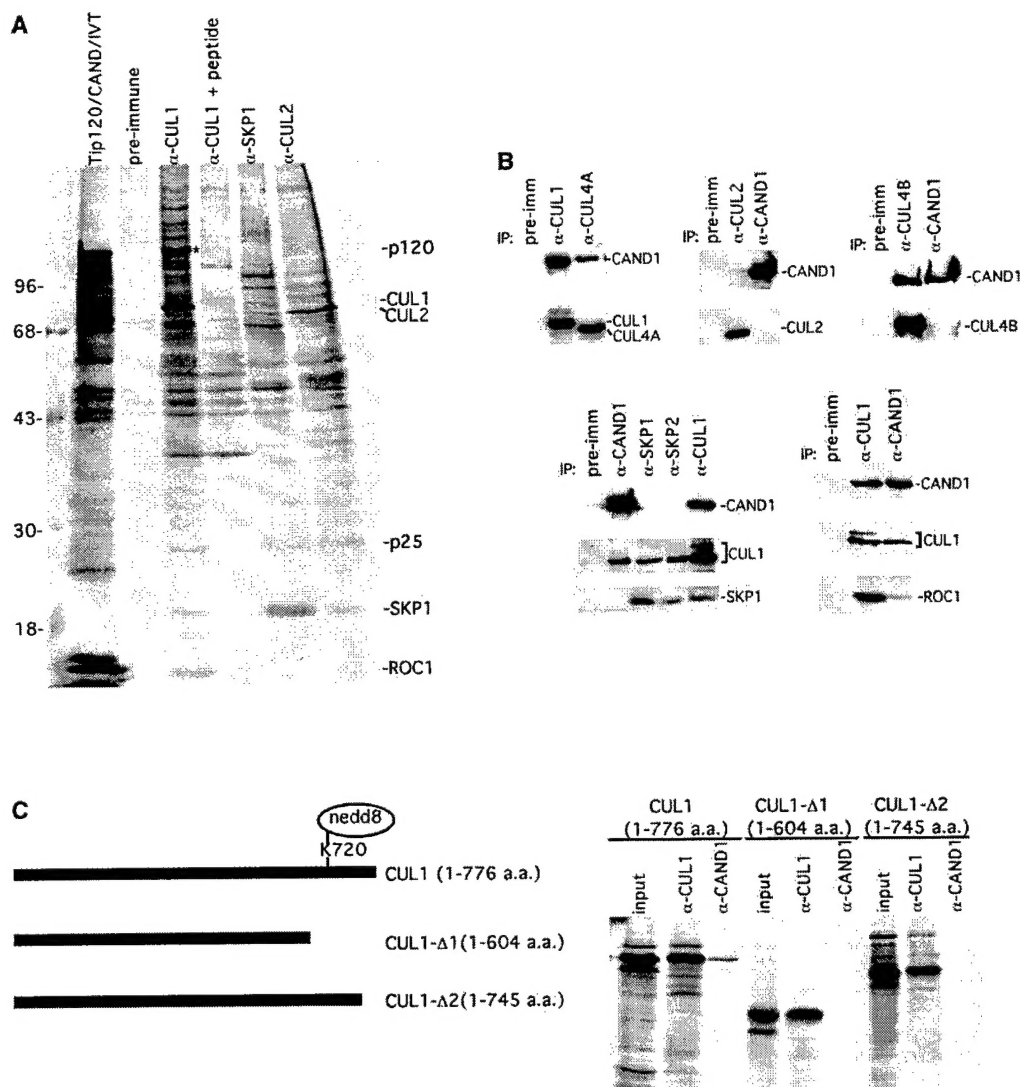[4]Correspondence: hui.zhang@yale.edu

Figure 1. p120^CAND1 Binds to Cullins and ROC1

(A) ³⁵S-methionine-labeled HeLa lysates were immunoprecipitated with preimmune, anti-CUL1 with or without a competing peptide, SKP1, or CUL2 antibodies. The in vitro-translated (IVT) Tip120A/CAND1 was included as a marker.

(B) CAND1 interacts with CUL1, CUL-4, and ROC1 but not with SKP1 and SKP2. HeLa lysates were immunoprecipitated (IP) with antibodies against various cullins, and SKP1, SKP2, and CAND1 antibodies. They were blotted with respective antibodies as indicated on the right of each panel.

(C) CAND1 binding to CUL1 requires the carboxyl terminus of CUL1. (Left) Diagram for CUL1 deletion mutants. (Right) ³⁵S-labeled CUL1 and its deletion mutants were incubated with HeLa extracts and then immunoprecipitated with anti-CUL1 or CAND1 antibodies.

The recombinant human TIP120A was found to electrophorese at the same mobility as p120 (Figure 1A). Partial V8 protease mapping of the recombinant TIP120A against p120 also suggested that they were identical (data not shown). We found that TIP120A strongly interacted with CUL1 and CUL4A and 4B, and weakly associated with CUL2 (Figure 1B). Because TIP120A is a major cullin binding protein, we renamed it CAND1 for cullin-associated nedd8-dissociated protein 1 (see below). We have also noticed the existence of a TIP120A homolog, TIP120B, in the EST and the GenBank databases (Aoki et al., 1999). The close homology between TIP120A and B suggests that TIP120B might also be a cullin binding protein. We would like to name TIP120B as CAND2.

CAND1 Is Present in a Complex with CUL1 and ROC1 but Does Not Bind to SKP1 and SKP2

The active SCF ubiquitin E3 ligase complex contains SKP1, CUL1, F box proteins such as SKP2, and ROC1 (Deshaies, 1999). Analysis of immunoprecipitated SCF and CAND1 complexes revealed that although SKP1 and SKP2 strongly interacted with CUL1, there was little association between CAND1 and SKP1 or SKP2 (Figure 1B). However, ROC1 was found to associate with CAND1 (Figure 1B), albeit to a lesser extent than CUL1. These analyses suggest that CAND1 forms a complex with CUL1 and ROC1 independently of SKP1 and SKP2.

It has been shown that CUL1 serves as a scaffold protein to form the active SCF complexes (Wu et al., 2000). ROC1 binds to its conserved carboxyl terminus

to couple SCF to the ubiquitin conjugation enzymes while SKP1 interacts with the amino terminal region of CUL1 and the F box proteins. SKP1 thus acts as an adaptor between the CUL1/ROC1 complex and the F box proteins. The F box proteins interact with substrates and provide the substrate specificity of various SCF complexes (Bai et al., 1996).

To determine the binding requirement for CAND1, we generated CUL1 truncation mutants. In vitro-translated and ^{35}S-methionine-labeled CUL1 and its mutants were incubated with HeLa extracts, and their binding to CAND1 was analyzed. While CUL1 clearly interacted with CAND1 (Figure 1D), deletion of just 31 amino acids from the carboxyl terminus of CUL1 abolished CAND1 binding (Figure 1D), suggesting that CAND1 association requires the carboxyl terminus of CUL1. The carboxyl termini of cullins are well conserved (Deshaies, 1999), raising the possibility that this conserved domain in cullins may provide a binding or recognition site for CAND1 or other CAND1-like proteins such as CAND2.

CAND1 Preferentially Binds to Unneddylated CUL1

In vivo, a small fraction of CUL1 is neddylated at lysine 720 at the carboxyl terminal region. This covalent modification retards the mobility of cullins during electrophoresis (Podust et al., 2000). Neddylation appears to associate with the active SCF or other cullin-containing E3 ligases (Ohh et al., 2002; Podust et al., 2000; Read et al., 2000). We have observed that CAND1-associated CUL1 usually corresponded to its unmodified form (Figures 1B and 2A). Since both neddylation and CAND1 require the carboxyl end of CUL1, we speculated that CAND1 binding might be modulated by the neddylation status of CUL1.

To test whether neddylation of CUL1 regulates CAND1 binding, we used HeLa cytosolic extracts to modulate the neddylation status of CUL1. Neddylation of cullins is mediated by NEDD8 activating E1 and conjugating E2 enzymes in an ATP-dependent process (Hochstrasser, 2000). Deneddylation is carried out by CSN independently of ATP (Lyapina et al., 2001). We found that neddylation of CUL1 is dynamically regulated in HeLa extract. Incubation of the extract with purified porcine CSN complex led to the deneddylation of CUL1 (Figure 2A). However, incubation of the extract with a polyclonal antibody against CSN2, a component of CSN, resulted in the conversion of most of CUL1 into the neddylated form (Figures 2B and 2C). This antibody probably blocks the access of endogenous CSN to CUL1, preventing CUL1 deneddylation. Conversely, removal of endogenous ATP by apyrase converts most of CUL1 into the unneddylated form (Figures 2B and 2C). This reaction is likely catalyzed by endogenous CSN in the extract.

Using this system, we found that CAND1 preferentially associated with the unneddylated form of CUL1 (Figures 2B and 2C). Recombinant GST-CAND1 preferentially pulled down the unneddylated form of CUL1 from the extract (Figure 2B). Because the CSN2 antibody shifts the equilibrium to promote neddylation of CUL1, it markedly reduced the binding of CAND1 to CUL1 in the extract (Figures 2B and 2C). The endogenous CAND1 in the extract exhibited the same preference as the exogenous CAND1 for the unmodified CUL1 (Figure 2C). Con-

versely, apyrase treatment reduced the neddylation of CUL1 and enhanced the interaction between CAND1 and CUL1 (Figures 2B and 2C). These results suggest that CAND1 requires the carboxyl terminus of CUL1 for binding and neddylation of lysine 720 in CUL1 interferes with the interaction between CAND1 and CUL1.

Binding of CAND1 to CUL1 Prevents the Interaction between SKP1/SKP2 and CUL1, and Inhibits p27 Polyubiquitination in HeLa Extract

CAND1 binding to CUL1 appears to be independent of SKP1 and SKP2 (Figure 1B). To determine whether CAND1 affects the binding of SKP1 or SKP2 to CUL1, recombinant GST or GST-CAND1 was added to HeLa extracts. While GST did not affect SKP1 binding, GST-CAND1 caused a marked dissociation of both SKP2 and SKP1 from CUL1 in the extract (Figure 2D). These data indicate that CAND1 binding to CUL1 prevents the interaction between SKP1/SKP2 and CUL1, suggesting that the binding of CAND1 or SKP1/SKP2 to CUL1 is mutually exclusive.

To examine whether CAND1 can inhibit SCF activity, we examined the effect of CAND1 on p27 ubiquitination. We have previously established a HeLa cytosolic extract that recapitulates the SCF^{SKP2}-mediated p27 ubiquitination and subsequent proteolysis (Tsvetkov et al., 1999). We found that addition of GST-CAND1 but not the same amount of GST inhibited p27 ubiquitination (Figure 2E). Thus, the binding of CAND1 to CUL1 can inhibit p27 ubiquitination, likely through the dissociation of SKP1 and SKP2 from the SCF complex.

Assembly of the SCF Complex in HeLa Extract Requires ATP and Neddylation of CUL1

If CAND1 association with CUL1 is inhibitory for SKP1/SKP2 binding, its dissociation following neddylation of CUL1 may conversely promote the binding of SKP1 and SKP2 to CUL1. To determine this possibility, CUL1 neddylation was modulated by varying ATP levels in the extract (Figure 3A). Removal of ATP by apyrase again reduced CUL1 neddylation and enhanced CAND1 association with CUL1 (Figure 3A, lane 2). An increased association of ROC1 with CUL1 was also observed (Figure 3A, lane 2). In contrast, removal of ATP induced SKP1 and SKP2 dissociation from CUL1 (Figure 3A, compare lanes 1 and 2). Conversely, addition of ATP in the extract markedly promoted CUL1 neddylation. Interestingly, while increasing CUL1 neddylation by the addition of ATP caused dissociation of CAND1, this process greatly stimulated the association between CUL1 and SKP1 or SKP2 (Figure 3A, lanes 3–7). Although ROC1 association with CUL1 appeared to be higher without ATP, increasing ATP and neddylation of CUL1 led to a slight reduction in their association (Figure 3A). Addition of ADP or AMP-PNP produced no effect (data not shown).

We also examined the effect of a low concentration (2 mM) of ATP and the presence of anti-CSN2 antibody which promotes neddylation (Figure 3B). In this case, both neddylation of CUL1 and SKP1 binding were again greatly enhanced, which correlated with the dissociation of CAND1 from CUL1. Our results suggest that in the presence of ATP, neddylation of CUL1 and removal of CAND1 are coupled to the association of SKP1 and
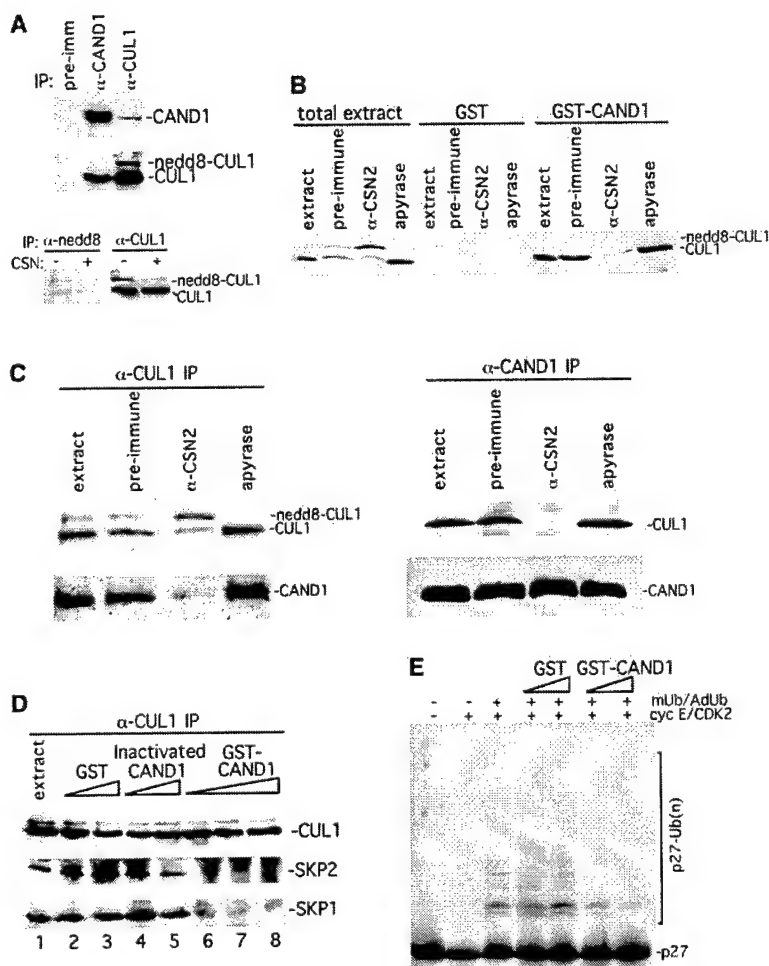
Figure 2. CAND1 Preferentially Binds to the Unneddylated Form of CUL1 and Blocks SKP1 and SKP2 Binding to CUL1

(A) (Top) CUL1 and CAND1 immunoprecipitates from IMR90 fibroblasts were blotted with anti-CAND1 and CUL1 antibodies. (Bottom) 1.5 μg of purified CSN was incubated with 100 μg of HeLa extract for 30 min. The CUL1 immunoprecipitates were blotted with anti-nedd8 (left) or CUL1 (right) antibodies.

(B) HeLa extracts (100 μg) were incubated with either anti-CSN2 antibodies in the presence of 3 mM ATP or 100 units/ml apyrase for 1 hr. Two micrograms of GST or GST-CAND1 was then added, incubated, and pulled down with glutathione beads and blotted with anti-CUL1 antibodies.

(C) Extracts were treated with CSN2 antibodies or apyrase. CUL1 and CAND1 complexes were immunoprecipitated from HeLa extracts and blotted with either anti-CUL1 (top row) or CAND1 (bottom row) antibodies.

(D) CAND1 prevents SKP1 and SKP2 association with CUL1. One hundred micrograms of HeLa extracts was incubated with either 2 and 5 μg of GST, the heat-inactivated GST-CAND1, or 1, 2, and 5 μg of GST-CAND1 for 60 min. The CUL1 complexes were immunoprecipitated and blotted with anti-CUL1, SKP2, or SKP1 antibodies.

(E) CAND1 inhibits p27 ubiquitination in HeLa extracts. Two hundred micrograms of HeLa extracts was incubated with GST (0.5 and 2 μg) or GST-CAND1 (0.5 and 2 μg) for 30 min. ^{35}S-labeled p27, cyclin E/CDK2, and ubiquitin-aldehyde (AdUb) and methylated ubiquitin (mUb) were added and were incubated for 2 hr. p27 was immunoprecipitated and visualized by fluorography.

SKP2 with the CUL1/ROC1 complex, leading to assembly of the SCF^{SKP2} complex.

SKP1 and ATP Cooperatively Mediate CAND1 Dissociation from CUL1

To further investigate the effect of neddylation on CUL1, a CUL1 mutant was made in which the critical lysine 720 for neddylation is converted to arginine (K720R). This mutation was shown to abolish CUL1 neddylation (Read et al., 2000). Indeed, the K720R mutant could not be neddylated in our HeLa extract (Figure 3C). In addition, the ability of CAND1 to interact with the K720R mutant appeared to be much lower than the wild-type CUL1, suggesting that CAND1 may recognize the conserved lysine 720 in CUL1 for its binding (Figure 3C). However, we found that addition of ATP still reduced the binding of CAND1 to the K720R mutant in the extract (Figure 3C, lane 6), suggesting that ATP can regulate the interaction between CAND1 and CUL1 independently of CUL1 neddylation.

The association of CAND1 and SKP1 with CUL1 appears to be mutually exclusive (Figure 2D). To determine whether SKP1 or SKP2 also conversely affects CAND1 binding to CUL1, the CAND1/CUL1 complex was isolated from HeLa extract pretreated with apyrase (Figure 3D). This complex was then incubated with either puri-

fied GST, GST-SKP1, or GST-SKP2 with or without ATP (Figure 3D). CUL1 complexes were then repurified and CAND1 binding to CUL1 was determined. We found that while either SKP1 or ATP alone did not significantly affect CAND1 association with CUL1, SKP1 and ATP act cooperatively to dissociate CAND1 from CUL1 (Figure 3D). This dissociation of CAND1 occurs without CUL1 neddylation, as the original CAND1/CUL1 complex was isolated from the extract in which ATP was removed. However, incubation of SKP2 did not appear to cause CAND1 dissociation from CUL1 (Figure 3D). Thus, either neddylation of CUL1, or SKP1 and ATP can regulate CAND1 binding to CUL1. Regulation of the concentration of these factors may modulate the assembly of the SCF complex.

Overexpression of CAND1 Causes the Dissociation of SKP1 from CUL1 but Promotes ROC1 Binding

The in vivo function of CAND1 was initially investigated by ectopic expression of CAND1. Overexpression of CAND1 in Phenix cells led to its strong association with CUL1 (Figure 4A). Consistent with our in vitro data (Figure 2D), expression of CAND1 also induced the significant dissociation of the endogenous SKP1 and SKP2 from CUL1 (Figure 4A and data not shown), suggesting
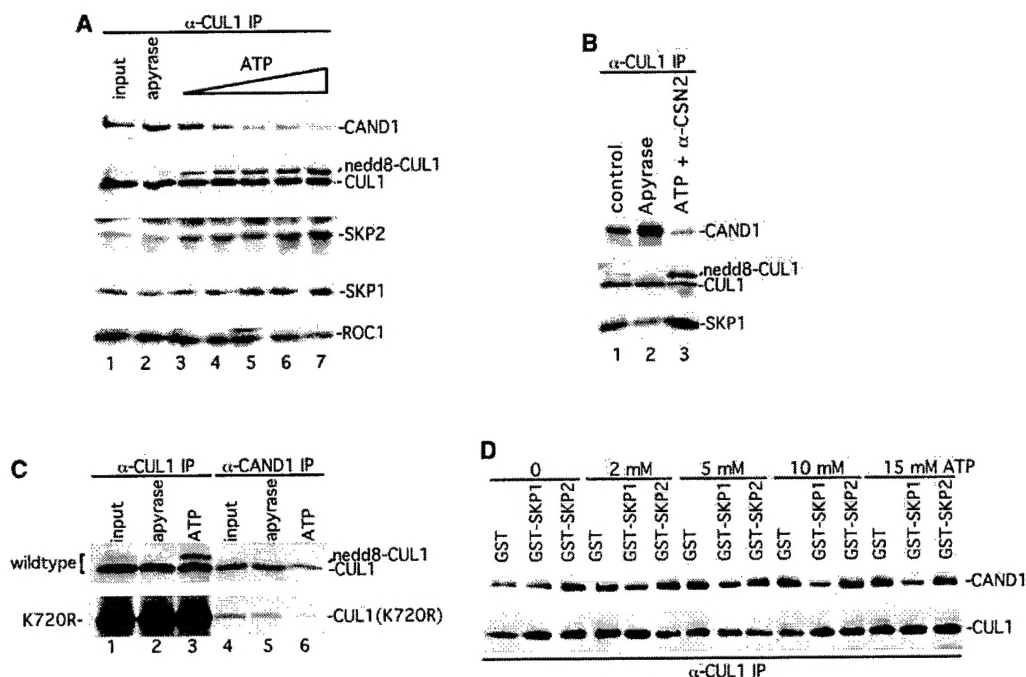
Figure 3. Neddylation and ATP Cause CAND1 Dissociation and Promote SKP1 and SKP2 Binding to CUL1

(A) HeLa extracts were treated with apyrase or increasing concentrations of ATP (2, 5, 7.5, 10, and 15 mM) for 30 min. The CUL1 complexes were immunoprecipitated and blotted with anti-CAND1, CUL1, SKP2, SKP1, or ROC1 antibodies.

(B) HeLa extracts were treated either with apyrase or 2 mM ATP plus anti-CSN2 antibody with an ATP regenerating system. The CUL1 immunoprecipitates were blotted with anti-CAND1, CUL1, or SKP1 antibodies.

(C) ATP prevents the binding of CAND1 to CUL1 in HeLa extracts. The ^{35}S-labeled CUL1 and the K720R mutant were incubated with extracts in the presence of apyrase or 10 mM ATP and then immunoprecipitated with anti-CUL1 or CAND1 antibodies.

(D) ATP and SKP1 cooperate to dissociate CAND1 from the CUL1 complex independently of neddylation. The CAND1/CUL1 complexes were immunoprecipitated by CUL1 antibodies from the apyrase-treated extract. They were then incubated with 1 μg GST, GST-SKP1, or GST-SKP2 with or without ATP for 30 min. The CUL1 complex was reisolated and blotted with anti-CAND1 or CUL1 antibodies.

that CAND1 can be inhibitory in vivo for the formation of the SCF complex.

We have also repeatedly observed that although the endogenous SKP1 and SKP2 dissociated from CUL1 in CAND1-expressing cells, the interaction between endogenous ROC1 and CUL1 was in fact slightly stimulated by CAND1 (Figure 4A). Such an increase is consistent with our finding that CAND1 interacts with both CUL1 and ROC1 (Figure 1C) and that in the apyrase-treated extract, the binding of CAND1 and ROC1 to CUL1 increases in parallel with reduced SKP1 or SKP2 association (Figure 3A).
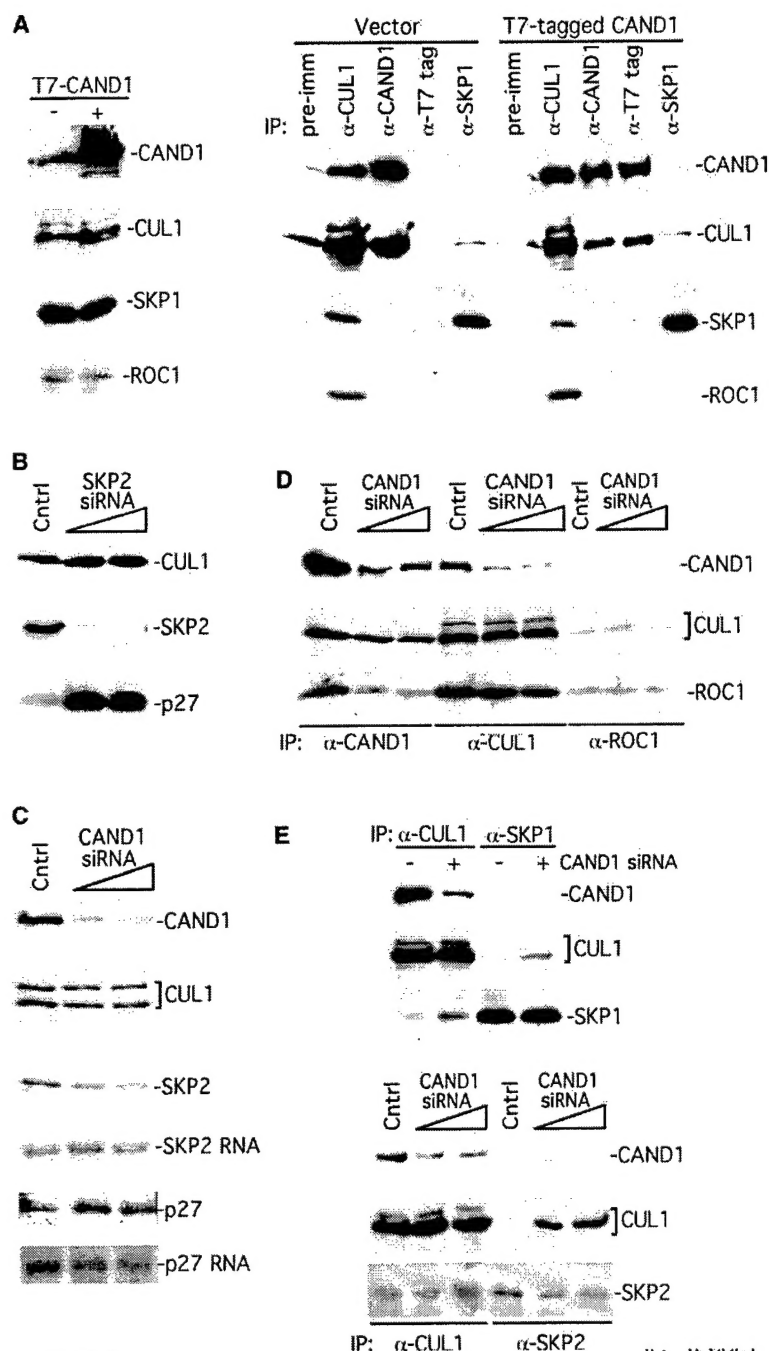
Effect of Silencing CAND1 in Human Cells

To further analyze the function of CAND1 in vivo, the expression of endogenous CAND1 was silenced by siRNA (Elbashir et al., 2001). To determine the efficiency of siRNA, a SKP2 siRNA was initially tested. Treatment of HeLa cells with SKP2 siRNA but not a control siRNA caused significant downregulation of SKP2 (Figure 4B). To determine whether the siRNA effect of SKP2 is functional, the levels of p27, a critical SKP2 substrate, were monitored. As expected, silencing of SKP2 greatly induced p27 levels (Figure 4B).

We found that incubation of HeLa cells with siRNA against CAND1 significantly reduced CAND1 protein levels and its interaction with CUL1 (70%–90% efficiency, various between experiments) within 48 hr (Figures 4C–

4E). Since we found that in HeLa extract dissociation of CAND1 removed an inhibitory effect on the binding of SKP1 and SKP2 to CUL1 and caused the enhanced interaction between SKP1/SKP2 and CUL1 (Figures 3A and 3B), we examined whether CAND1 has the same effect in vivo. HeLa cells were treated with siRNA against CAND1 to reduce its levels. CUL1, SKP1, and SKP2 containing complexes were then immunoprecipitated from control and CAND1 siRNA-treated cells. The presence of these proteins in the SCF complex was analyzed and compared. We have consistently observed that, in parallel to the disappearance of CAND1 from CUL1 by CAND1 siRNA, loss of CAND1 resulted in a significant increase of the binding of both SKP1 and SKP2 to CUL1 (Figure 4E). These results indicated that both in vivo and in vitro, CAND1 binding to CUL1 negatively regulates the association between SKP1 and F box protein SKP2 and CUL1.

We also observed that knockdown of CAND1 levels often caused a small reduction in total SKP2 levels (Figures 4C and 4E). Previous studies suggest that F box proteins such as SKP2 are not stable; they undergo a CUL1-dependent and ubiquitin-mediated proteolysis (autoubiquitination) (Wirbelauer et al., 2000; Zhou and Howley, 1998). It is possible that in CAND1-silenced cells enhanced binding of SKP2 to CUL1 promotes SKP2 autoubiquitination, leading to its subsequent proteolysis. Consistent with this possibility, we found that down-

A

T7-CAND1

Vector   T7-tagged CAND1

IP: pre-imm α-CUL1 α-CAND1 α-T7 tag α-SKP1   pre-imm α-CUL1 α-CAND1 α-T7 tag α-SKP1

-CAND1
-CUL1
-SKP1
-ROC1

B

Cntrl SKP2 siRNA

-CUL1
-SKP2
-p27

C

Cntrl CAND1 siRNA

-CAND1
]CUL1
-SKP2
-SKP2 RNA
-p27
-p27 RNA

D

Cntrl CAND1 siRNA   Cntrl CAND1 siRNA   Cntrl CAND1 siRNA

-CAND1
]CUL1
-ROC1

IP: α-CAND1   α-CUL1   α-ROC1

E

IP: α-CUL1 α-SKP1

- + - + CAND1 siRNA

-CAND1
]CUL1
-SKP1

Cntrl CAND1 siRNA   Cntrl CAND1 siRNA

-CAND1
]CUL1
-SKP2

IP: α-CUL1   α-SKP2

Figure 4. CAND1 Regulates SKP1 and SKP2 Binding to CUL1 In Vivo

(A) Expression of CAND1 prevents SKP1 binding to CUL1. Phenix cells were transfected with the vector or the vector encoding T7 epitope-tagged CAND1 for 48 hr. The total levels of CAND1 and components of SCF were analyzed (left panel). Equal lysates (300 µg proteins) were immunoprecipitated with various antibodies as indicated and blotted with anti-CAND1, CUL1, SKP1, or ROC1 antibodies (right panel).
(B) Silencing of SKP2 causes p27 accumulation. HeLa cells were incubated with siRNAs for luciferase (control, 150 nM) and SKP2 (50 and 150 nM) for 48 hr. The levels of CUL1, SKP2, and p27 were blotted.
(C–E) Silencing of CAND1 in HeLa cells caused increased binding of SKP1 and SKP2 to CUL1 and downregulation of SKP2. HeLa cells were treated with either control or CAND1 siRNA (50 and 150 nM, except 150 nM was used for SKP1 binding in [E] for 48 hr). (C) The protein levels of CAND1, CUL1, SKP2, and p27 in control and CAND1 siRNA-treated cells were determined by Western blot. In parallel, the RNA levels of SKP2 or p27 were analyzed by Northern blot. (D) The CAND1, CUL1, and ROC1 immunoprecipitates from control and CAND1 siRNA-treated cells were blotted with anti-CAND1, CUL1, or ROC1 antibodies. (E) The association of CUL1 with SKP1 (top) and SKP2 (bottom) in the control and CAND1 siRNA-treated cells was analyzed by immunoprecipitation followed by Western blot as indicated.

regulation of SKP2 in CAND1-deficient cells occurs only at the protein level, as the SKP2 RNA level does not appear to decrease (Figure 4C). However, we could not successfully measure alterations in the half-life of SKP2 because of the small change in SKP2 protein levels. In the CAND1 siRNA-treated cells, a slight increase in p27 levels was also observed (Figure 4C). This increase of p27 occurred at the protein level since there was no corresponding increase in p27 RNA (Figure 4C). These observations are consistent with results from previous studies showing that incorporation of F box proteins into

the SKP1/CUL1 complex leads to their destabilization (Wirbelauer et al., 2000; Zhou and Howley, 1998).

CAND1 binds to both CUL1 and ROC1 (Figure 1B). In cells overexpressing CAND1, the binding of ROC1 to CUL1 appears to increase (Figure 4A). To determine the silencing effect of CAND1, the interaction between CUL1 and ROC1 was examined in control and CAND1 siRNA-treated cells. Our studies suggest that CAND1 silencing caused a slight decrease in the interaction between ROC1 and CUL1 (Figure 4D). However, the majority of ROC1 is still bound to CUL1 in CAND1-deficient cells.

These data suggest that CAND1 may contribute to modulation of the interaction between ROC1 and CUL1.

Discussion

We have isolated p120^{CAND1} as a CUL1 binding protein and found that it also binds to ROC1 but not to SKP1 and F box protein SKP2 (Figures 1 and 4). Both in vitro and in vivo, CAND1 regulates the formation of the SCF complex by preventing the association between CUL1 and SKP1/SKP2 (Figures 2D and 4A). Dissociation of CAND1 from CUL1 is regulated by neddylation of CUL1 (Figure 2) or the presence of SKP1 and ATP independent of neddylation (Figure 3). The dissociation of CAND1 from CUL1 is tightly coupled to the binding of SKP1 and F box proteins to CUL1 (Figures 3A, 3B, and 4E). Our studies thus suggest that CAND1 binding to CUL1 provides a mechanism for the regulation of the SCF ubiquitin E3 ligase complex.

The neddylation pathway is highly conserved and is essential in many organisms (Hochstrasser, 2000). Studies using the cell extract system have shown that neddylation of cullins is required for the polyubiquitination of p27^{KIP1}, IκB, or HIF (Ohh et al., 2002; Podust et al., 2000; Read et al., 2000). However, since the complete ubiquitination of p27 can be reconstituted by purified recombinant proteins in vitro, it is not apparent why neddylation of CUL1 is essential (Spruck et al., 2001; Tsvetkov et al., 1999). The requirement for neddylation suggests that in the HeLa extract an activity might exist that inhibits the SCF function if CUL1 is deneddylated. Since CAND1 only interacts with unneddylated CUL1 (Figure 2) and this interaction inhibits the SCF formation, it is possible that in the previous reports lack of CUL1 neddylation promotes the binding of CAND1 to CUL1, thereby preventing the association of SKP1 and F box proteins with CUL1 and causing the inhibition of p27 polyubiquitination. Since CAND1 strongly binds to CUL4 (Figure 1B), it is possible that CAND1 or CAND1-like activity may also regulate other cullin activities.

It has been shown that F box proteins are short-lived proteins which are destabilized by their incorporation into the SCF complex (Wirbelauer et al., 2000; Zhou and Howley, 1998). Our data suggest that CAND1 regulates the formation of the SCF complex by preventing the binding of F box proteins and SKP1 to CUL1. One consequence of the loss of CAND1 and enhanced SKP2 and SKP1 binding to CUL1 is the downregulation of SKP2 (Figure 4E). Our observations are consistent with the previous reports that binding of F box protein to CUL1 facilitates their own destruction and, by preventing F box protein binding to CUL1, CAND1 binding to CUL1 may help promote the stability of F box protein SKP2. In addition, because CUL1 has to recycle between various SCF complexes, CAND1 binding may also promote the recycling of CUL1 during the catalytic ubiquitination cycle.

So far we only find close CAND1 homologs in the databases of *Arabidopsis thaliana* (hypothetical protein T8K22.14, accession number T00607), *Caenorhabditis elegans* (hypothetical protein Y102A5A.1, accession number T25024), *Drosophila melanogaster* (CG5366/ GH07774), and mammals. The existence of CAND1-like activities in other organisms requires further examination. Since neddylation of cullins is highly conserved, it remains to be determined whether neddylation has further functions. We also cannot rule out that CAND1 has additional functions (Yogosawa et al., 1996). Further studies are necessary to elucidate the regulation of cullin E3 ligases in many important biological processes.

Experimental Procedures

Cells and Proteins
HeLa, 293, Phenix, and IMR90 cells were cultured as described before (Zhang et al., 1995). The porcine COP9 signalosome (CSN) was purified as described previously (Wei and Deng, 1998). All GST proteins were purified from bacteria.

Purification of p120 and Cloning of Its Encoding cDNA
Suspension HeLa cells (50 liters) were used for purification of p120 using anti-CUL1 immunoaffinity column (Zhang et al., 1995). Two derivative peptides were obtained: p120K24: (S)VILEAFSSPSEEVK and p120K25: F(X)I(D)DHPQPIDD(L)(L)K. Both matched to rat TIP120A and human KIAA0667. The full-length human CAND1 cDNA was isolated from a HeLa λ phage cDNA library (Stratagene) and sequenced entirely for confirmation (Zhang et al., 1995).

Immunological Reagents and Procedures
The anti-CAND1, CUL2, CUL4A, NEDD8, CUL4B, and ROC1 antibodies were raised as described in the supplemental data at http:// www.molecule.org/cgi/content/full/10/6/1519/DC1. The anti-CSN2, SKP2, SKP1, p27, and CUL1 antibodies were used as described (Tsvetkov et al., 1999; Wei and Deng, 1998).

Cloning and Expression
The truncation mutants of CUL1 were made by restriction enzymes with either Sal I (CUL1-Δ1) or Xho I (CUL1-Δ2). The CUL1 K720R mutant was generated by the site-directed mutagenesis using the Stratagene protocol. They were in vitro translated and ^{35}S-methionine labeled.

Extracts, p27 Ubiquitination, and Northern Blots
Cytosolic HeLa extracts for CUL1 neddylation, CAND1 binding, and p27 ubiquitination were conducted at 30°C as described previously (Tsvetkov et al., 1999). For Northern blots, equal amounts of RNA (5 μg) were used.

RNA Interference/Silencing in HeLa Cells
The siRNA-mediated gene silencing (Elbashir et al., 2001) using SKP2 or CAND1 siRNA was conducted in HeLa cells as described in the supplemental data at http://www.molecule.org/cgi/content/ full/10/6/1519/DC1.

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